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A putative effector of Ral has homology to Rho/Rac GTPase activating proteins

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We report here the cloning of a gene encoding a novel Ral interacting protein (RIP1) from a cDNA expression library using radiolabeled Ral as probe. RIP1 binds to Ral in a GTP-dependent manner. The 4.1 kb transcript of the RIP1 gene is present in all tissues analysed and encodes for a protein product of 648 residues. RIP1 shares sequence similarity with GAP proteins that are capable of activating the GTPase activity of members of the Rho/Rac family of GTPases. When tested, RIP1 could activate the GTPase activity of CDC42 and, to a lesser extent, Rac1 but not RhoA, Ras, or Ral. Activated Ral had no direct effect on the GTPase-activating ability of RIP1, *in vitro*.

Keywords: Ral; Rho GTPase activating protein; protein interactions

Introduction

The Ral protein is one of a large family of low molecular weight GTPases, the most well-known of which is Ras. Like other members of this family of proteins, Ras functions as a molecular switch, alternating between a GTP bound 'on' state and a GDP-bound 'off' state. The association of Ras with these nucleotides is regulated by two different types of proteins. The guanine nucleotide exchange factors (GEFs) stimulate the exchange of GDP for GTP, thereby switching Ras to the 'on' state. Conversely, GTPase-activating proteins (GAPs) enhance the normally low, intrinsic GTPase activity of Ras, resulting in the hydrolysis of GTP or GDP and the associated switching of Ras to the 'off' state.

The Ras superfamily of proteins can be divided into four different subfamilies according to their sequences: Ras, Rho/Rac, Rab, and ARF (ADP ribosylation factor). This division also delineates function. Members of the Rho/Rac subfamily are involved in the regulation of the cytoskeleton, while members of the Rab family are involved in vesicular transport. Ral, along with Rap, are members of the Ras subfamily.

Ral is a 206 amino acid protein which shares greater than 50% homology with Ras. Ral transcripts are expressed in a wide variety of tissues, with the highest levels detected in testis and ovaries, somewhat less in brain, adrenal and pituitary glands, kidney and ovary and still less in muscle tissue (Olofsson *et al.*, 1988; Wildey *et al.*, 1993). The Ral proteins have been identified as being a major GTP binding protein in human platelets (Polakis *et al.*, 1989; Bhullar *et al.*, 1990) as well as being a major protein in the supernatant fraction of rabbit and bovine brains (Bhullar, 1992).

A GAP activity for Ral has previously been demonstrated in cytosolic fractions of brain and testis (Emkey *et al.*, 1991). The activity sediments between 150 and 443 kDa. The protein responsible for this Ral-GAP activity has yet to be identified.

A guanine nucleotide exchange factor specific for only RalA and RalB has been identified (Albright *et al.*, 1993) and named ralGDS for Ral guanine-nucleotide dissociation stimulator. ralGDS has been implicated as a possible effector for Ras through its association with activated Ras in yeast two-hybrid systems (Hofer *et al.*, 1994; Kikuchi *et al.*, 1994; Spaargaren *et al.*, 1994).

In an attempt to elucidate the role of Ral in signalling, we set out to identify proteins that would interact with Ral in a GTP-specific manner. To this end, we set up a screen to search for proteins that were capable of interacting physically with Ral.

Results

Isolation of the RIP1 clone

In order to detect proteins that were capable of physical interaction with Ral, a radiolabeled Ral protein was used as probe to screen a cDNA expression library. This method was chosen instead of the commonly used yeast two-hybrid system because the use of a protein probe made it possible to preload the probe with either GDP or GTP; this in turn made it possible to screen for interacting proteins that bound specifically to the GTP form of the Ral protein.

The probe used in this screen was a modified version of human RalB. In order to facilitate radiolabeling of this protein *in vitro*, arg-arg-asp-ser-val (K-domain), a substrate for cAMP-dependent protein kinase, was fused to the N-terminus of Ral (Hateboer *et al.*, 1995). In addition, a poly-histidine tag (Janknecht *et al.*, 1991) was added to the C-terminus for purification purposes. The resulting chimeric protein, termed KH-ral, was expressed in bacteria and purified over a nickel column (Hochuli *et al.*, 1987).

The guanine nucleotide-binding property of the chimeric KH-ral protein was found to be similar to that of previously purified, bacterially produced, human RalB. (data not shown; Albright *et al.*, 1993) We wanted to confirm that the effector domain of

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KH-ral also remained unaffected by the modifications undertaken to construct this probe. The effector domain was originally defined in the related Ras protein as the region in which mutations rendered oncogenic mutants of Ras no longer able to transform; at the same time, such mutations had no effect on the localization, GTPase activity or stability of the protein. Previously, we have found (B Giddings and R A Weinberg, unpublished) that the intactness of the corresponding region of the Ral protein, which we presume serves as the effector of domain of Ral, was required in order for ralGDS to stimulate guanine nucleotide exchange on Ral.

As a test of whether the effector region of KH-Ral remained functionally intact, nucleotide exchange reactions using purified ralGDS were conducted on this protein. Neither the presence of the extra N- and C-terminal residues sequence nor the phosphorylation of the K-domain caused a substantial difference in the ability of KH-ral to be recognized by ralGDS, indicating that the presence of the K-domain and the His-tag do not alter the structure of the effector domain of Ral.

KH-Ral was used to screen a cDNA expression library generated from day 10 mouse embryo cells. 2×10^5 plaques were screened with the radiolabeled, GTPγS preloaded KH-ral. One of the positive plaques, termed 19–6, was purified and its DNA insert of 1331 bases pairs was sequenced on both strands. This clone was named RIP1 (for *R*al *i*nteracting *p*rotein).

A 342 base pair EcoRI-PstI fragment subcloned from this cDNA clone was used as a DNA probe to screen the library for full length clones. Fourteen plaques that reacted with this probe were identified and divided into six groups by restriction mapping. Although none of the detected clones contained the entire sequence, overlapping sequences between them made it possible to deduce the full length sequence (Figure 1), which encompassed 3661 base pairs in which we found an open reading frame of 1944 bases encoding a protein of 648 amino acids. Approximately three kilobases including the open reading frame was sequenced on both strands, while the majority of the 3′ untranslated region was sequenced only on one strand.

Analysis of the imputed reading frame revealed a striking sequence homology shared with other proteins known to possess GTPase activating (GAP) activity toward members of the Rho/Rac sub-family of GTPases. (Figure 2) The homology extended over 147 amino acids. No significant homologies were identified in the domains flanking the rho GAP homology domain. The C-terminal domain was found

Figure 1 Nucleotide and deduced amino acid sequence of RIP1. The Rho/Rac GAP homology domain is underlined. The box indicates the Ral-binding region. The stop codon is indicated by an asterisk

to be rich in glutamic acid residues while the N-terminal domain was rich in both glutamic acid and lysine residues.

Nucleotide specificity

To test whether the binding of Ral to the RIP1 protein was dependent on the specific guanine nucleotide bound by Ral, we tested the relative affinities of the GDP- and GTP-loaded forms of the KH-ral probe for RIP1. Using the initially isolated phage, filters were prepared for the protein screen that contained induced 19–6 protein. Each filter was then cut in half and incubated with radiolabeled KH-ral that was preloaded with either GTPγS or GDP. As shown in Figure 3, 19–6 bound KH-ral strongly when it was preloaded with GTPγS, while only background level binding was detected when the KH-ral probe was preloaded with GDP. This indicated that the interaction between RIP1 and Ral was GTP-dependent, and thus provided strong support for the notion that RIP1 functions as an a effector of Ral.

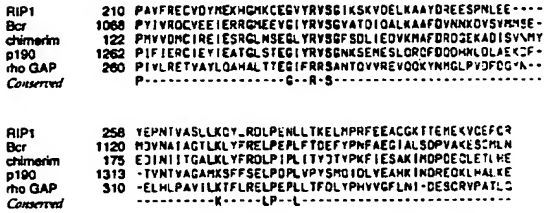

Figure 2 Amino acid sequence comparison of proteins containing Rho/Rac GAP activity. The sequence of RIP1 is compared to the sequence of Bcr (Diekmann *et al.*, 1991), chimerin (Diekmann *et al.*, 1991), p190 (Settleman *et al.*, 1992), and rhoGAP (Lancaster *et al.*, 1994). Amino acids present in 4–5 sequences are shown in bold. Conserved residues are shown as a consensus sequence

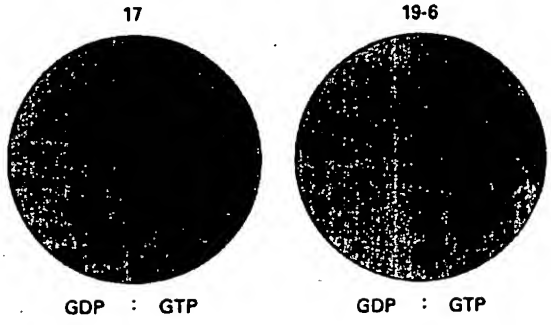

Figure 3 Preferential binding of 19–6 to KH-ral preloaded with GTP *vs* GDP. Filters were prepared as described in Material and methods and cut in half. The left half was hybridized to KH-ral probe preloaded with GDP, the right half was hybridized to KH-ral probe preloaded with GTPγS. 17 is a negative control

Interacting domain mapping

We wished to localize the region in RIP1 involved in its interaction with Ral. To do so, derivatives of the pEXlox19–6 plasmid encoding fragments of RIP1 were generated. Two series of nested deletions were constructed, one beginning from the C-terminus, the other from an internal Eco47 III site. The truncated RIP1 proteins encoded by these deleted forms of pEXlox19–6 were then synthesized by *in vitro* translation and incubated with HA (hemagglutinin antigen)-tagged human RalB protein that had been preloaded with GTPγS. Anti-HA antibody was used to immunoprecipitate any Ral:19–6 complexes that formed (Figure 4).

It was clear that the ability of RIP1 to bind Ral was critically dependent upon a domain that lay between 390 and 445. This segment is 34 amino acids C-terminal of the domain containing homology to Rho GAPs. We concluded that the observed binding was attributable to the interaction of Ral with a specific, well-defined domain of RIP1.

Tissue-specific expression of RIP1

We wished to determine the range of expression of the RIP1 gene in various tissues. To this end, we used a 342 base pair EcoRI-PstI fragment of RIP1 to probe two panels of Northern blots containing RNA from a total of 14 different human tissues (Figure 5). Although the levels of expression varied substantially, a 4.1 kilobase transcript was evident in all of the tissues examined. The highest level of expression was observed in ovaries and skeletal muscle, whereas the lowest was found in spleen, liver and peripheral blood leukocytes. Interestingly, these expression patterns did not resemble those of the Ral mRNA in these various tissues (Olofsson *et al.*, 1988; Wildey *et al.*, 1993).

The pattern of RIP1 protein expression was also examined. To this end, the C-terminal portion (see Material and methods) of RIP1 was expressed in an *E. coli* expression system and then purified; the resulting protein was used to generate anti-RIP1 antibodies in rabbits. The resulting unfractionated polyclonal sera failed to detect specific protein species in both

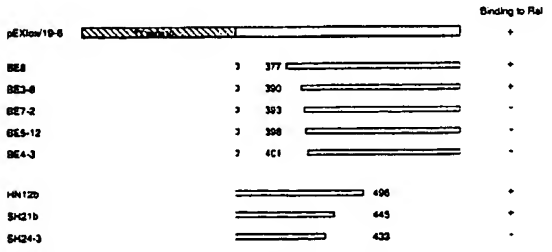

Figure 4 Mapping of the Ral-binding region. Two series of deletions were constructed and translated *in vitro*. The translates were incubated with HA-tagged human RalB preloaded GTPγS and immunoprecipitated with anti-HA antibody. The numbers indicate the position of the deletion. The hashed bar indicates the T7 gene 10 fusion protein of pEXlox

immunoprecipitation and Western blot experiments. However, affinity purification against full length RIP1 protein yielded an antibody which, when used as a probe in Western blot analysis, detected a 110 kilodalton species in lysates of Balb/3T3 cells (Figure 6).

We compared this size to that seen following direct expression of the cloned RIP1 gene. Introduction of the RIP1 coding segment into the pGEX-4T plasmid resulted in expression of a protein composed of RIP1 fused to glutathione S-transferase; this fusion protein could subsequently be cleaved with thrombin, releasing full length RIP1 protein. As an alternative, a segment containing the RIP reading frame was introduced into the pVL1393 baculovirus vector which allows expression in transfected insect cells.

While the amino acid sequence predicted a 75 kilodalton protein, a protein of approximately 110 kilodaltons was observed when bacterially produced protein was cleaved with thrombin. A protein of similar size was observed in the insect cells transfected with pVL1393. These sizes were virtually identical to those seen upon Western blotting of Balb/3T3 lysates, indicating that the cloned cDNA contains the entire reading frame of RIP1. The slight size discrepancy between the bacterially produced protein and the protein detected in Balb/3T3 lysates might be due to cleavage of RIP1 by thrombin at a potential cleavage site near the C-terminus or to the lack of proper post-translational modification (Figure 6).

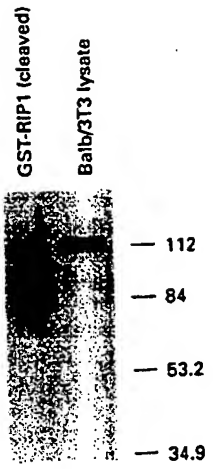

Figure 6 Western analysis of RIP1. Purified RIP1 cleaved from a GST fusion was run parallel to lysate prepared from Balb/3T3 cells. RIP1 was detected using affinity purified anti-RIP1 rabbit serum. The numbers indicate molecular weight in kilodaltons

GAP assays

To ascertain whether the Rho/Rac GAP homology domain found in RIP1 was indeed capable of activating the GTPase activity of the Rho, Rac, or related proteins, full length, recombinant RIP1 protein was used in GAP assays with either Rac1, CDC42Hs, RhoA, c-H-Ras, or RalB. RIP1 succeeded in stimulating the GTPase activity of Rac1 and CDC42. In the assay conditions used, 22% more of the GTP was hydrolyzed to GDP by Rac1 in the presence of RIP1, while as much as 35% was hydrolyzed by CDC42 in the presence of RIP1. However, no increase in GTPase activity could be detected when Ras or Ral were used in this assay, and very little, if any, could be detected when RhoA was used (Figure 7). While Ral interacts with RIP1 in a GTP-dependent manner, as shown above, this interaction had no effect on the intrinsic GTPase activity of Ral.

We also tested whether the binding of GTP-activated Ral to RIP1 affected the GAP activity of RIP1 toward Rac1, CDC42Hs or RhoA. To do this, RalB preloaded with either GTPγS or GDP was added to these GAP assays. Neither of these forms of Ral had an observable effect on the GAP activity of RIP1 toward these various G proteins (data not shown).

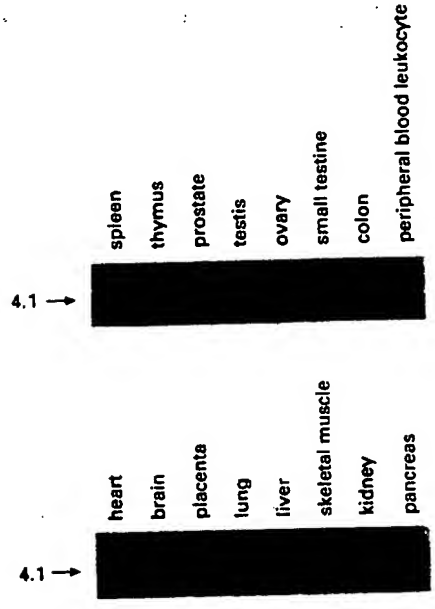

Figure 5 Tissue distribution of RIP1 transcript. A 342 base pair EcoRI-PstI fragment was used to probe multiple tissue Northern blots. Two micrograms of poly-(A) selected RNA from the indicated tissue was run in each lane. The number indicates size in kilobases

Discussion

Almost a decade has past since the isolation of the gene encoding Ral was first described (Chardin *et al.*, 1986). Although much work has been done in characterizing the biochemistry of the Ral protein and in cataloguing the various tissues in which it is expressed, the physiologic function of Ral has yet to be revealed. We describe here an attempt to elucidate the function of Ral through the identification of a putative effector protein.

A protein probe was used to screen directly a cDNA expression library for proteins that associated physically with Ral. A clone was identified encoding a protein, termed RIP1, that would interact with GTPγS-loaded Ral, which we presume to be the activated form

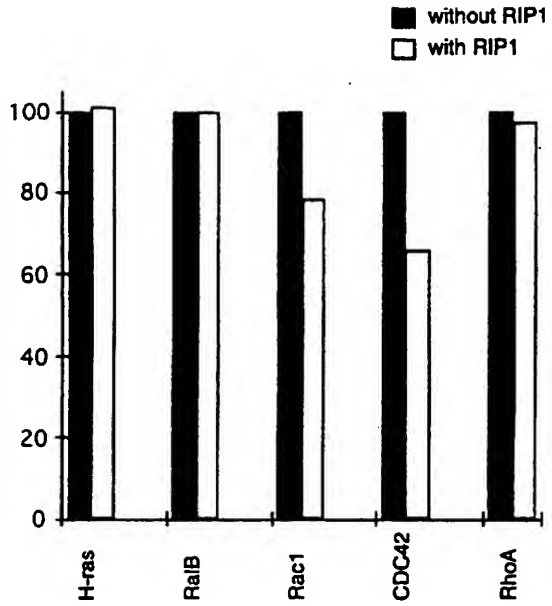

Figure 7 The effect of RIP1 on the GTPase activity of several GTPases. Ten ng of each GTPase was preloaded with [γ-^{32}P]GTP and incubated with or without 500 ng RIP1 for 10 min at 25°C. The products were separated by filtration and the filter-bound radioactivity was determined

of this protein. Importantly, the RIP1 protein clone would not interact with Ral when Ral was preloaded with GDP, underscoring the specificity of this interaction.

Of interest is the fact that RIP1 shares substantial sequence similarity with proteins known to exhibit GAP activity toward proteins in the Rho/Rac subfamily. Members of the Rho/Rac family have been implicated in cytoskeletal regulation. Thus, a strong induction of stress fibers was demonstrated when activated Rho protein was microinjected into serum-starved Swiss 3T3 fibroblasts (Ridley *et al.*, 1992a). Rac activation, in contrast, leads to the formation of lamellipodia, while CDC42 leads to the formation of filopodia in the same system (Ridley *et al.*, 1992b; Nobes *et al.*, 1995).

When recombinant RIP1 protein was purified and used in GAP assays, it was shown that this protein could activate the GTPase activity of CDC42 and, to a lesser extent, Rac1 but not that of RhoA, Ral, or Ras. While RIP1 interacts specifically with the GTP-bound form of Ral, this binding had no effect on the GTPase activity of Ral. Moreover, when the Ral-binding domain of RIP1 was mapped, it was found to be in a region C-terminal to, and not overlapping with, the GTPase activating domain of RIP1. This suggests that RIP1 does not serve as a regulator of Ral. Rather, this indicates that RIP1 is the target of activated Ral and thus serves as an effector of Ral.

The degree of GAP activity of RIP1 toward CDC42 and Rac1 is much lower than has been associated with other previously identified Rho/Rac GAP-containing proteins. This might be explained by the fact that the RIP1 protein may be labile, resulting in its substantial inactivation *in vitro*. Although several independent bacterial and insect cell preparations exhibited the same low specific GAP activity, it is possible nevertheless that one of the steps used commonly in all of the purifications caused the inactivation of much of the protein. Alternatively, it is possible that the RIP1 protein requires functional activation, either through covalent modification or the presence of an associated, distinct regulatory protein. Finally, it is possible that the *bona fide* target of RIP1 GAP activity has not yet been identified.

We considered the possibility that Ral might function as an allosteric regulator of RIP1, such that the binding of activated Ral to RIP1 would modulate the GAP activity of RIP1 toward its target GTPases. To test this possibility, GAP assays were done in the presence of excess GTPγS or GDP-loaded Ral. No modulation of RIP1 GAP activity toward RhoA, Rac1 or CDC42 was observed. This failure to observe a modulation of RIP1 GAP activity *in vitro* is not definitive.

We suggest, however, another mechanistic model of RIP1 action. Recent experiments have shown that activated Ras can bind directly to Raf and that recruitment of Raf to the membrane, where it is subsequently activated, is the essential role that Ras plays in the activation of Raf (Leevers *et al.*, 1994; Stokoe *et al.*, 1994). Thus, mutant forms of Raf that are targeted constitutively to the membrane are fully active in the absence of Ras. Analogously, Ral may serve to recruit RIP1 to a compartment in which it can function, or alternatively, to sequester RIP1, removing it from a compartment in which it serves as a GAP toward proteins like CDC42 or Rac.

A recent report by Cantor *et al.* describes a protein, RalBP1, that is likely to be identical to the protein described here (Cantor *et al.*, 1995). RalBP1 was isolated from a rat brain cDNA library using the yeast two-hybrid system. RalBP1 bound to wild type Ral but not to a Ral mutant in which residue 49 was mutated to asparagine. The corresponding mutation in Ras blocks its interaction with many of its downstream targets. The amino acid sequence of RalBP1 is 95.9% identical to RIP1. In experiments using recombinant RalBP1 GAP domain, RalBP1 was able to activate the GTPase activity of CDC42Hs to the highest degree. A slight activation was observed when Rac1 was used, while virtually none was detected when RhoA was used. A direct comparison between the activities RIP1 and RalBP1 cannot be made because only the GAP domain of RalBP1 was used and the amount used was not specified.

Materials and methods

Preparation and labeling of KH-ral

The human Ralb coding region was cloned into the KpnI-SacI site of pKH-E1a (Hateboer *et al.*, 1995), replacing E1a with Ral resulting in the expression of Ral upstream of six histidine codons and downstream of arginine-arginine-aspartate-serine-valine, a cAMP-dependent protein kinase phosphorylation site.

The plasmid was transformed into the BL21(DE3) strain of *E. coli* (Novagen). Following 1 h induction with 1 mM IPTG (Sigma) the bacteria were pelleted and resuspended in lysis buffer (50 mM TrisHCl, pH 8.0, 20% sucrose, 0.1 mM

EDTA, 0.1 mM EGTA, 10 mM beta-mercaptoethanol, 0.2 mM sodium-metabisulfite) plus protease inhibitors (1 mM PMSF, 5 $\mu g\ ml^{-1}$ aprotinin, 5 $\mu g\ ml^{-1}$ leupeptin) and sonicated. An equal volume of high salt buffer (50 mM TrisHCl, pH 8.0, 600 mM KCl, 10 mM beta-mercaptoethanol, 0.2 mM sodium-metabisulfite) was added to the sonicate and centrifuged. Ni-NTA resin (Qiagen) was added to the supernatant and washed with a 1:1 mix of lysis buffer and high salt buffer plus 1 mM imidazole (Sigma), then with PBS plus 16 mM imidazole. The protein was eluted in PBS plus 100 mM imidazole and dialyzed against PBS to remove the imidazole.

The purified protein was labeled *in vitro* by combining 100 μCi [γ-^{32}P]ATP (ICN), 30 units cAMP-dependent protein kinase (Sigma), and 1–2 μg protein to a final volume of 30 μl in protein kinase buffer (20 mM Tris pH 7.5, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT). Following incubation at 4°C for 30 min the probe was purified over a Sephadex G25 column prewashed with Tris NaCl (100 mM Tris pH 8.0, 120 mM NaCl) by eluting with Tris NaCl. KH-ral was then incubated in exchange buffer (50 mM Tris 7.5, 10 mM EDTA, 5 mM $MgCl_2$, 1 mg ml^{-1} BSA) with GTPγS (Sigma) for 20 min at 37°C. The reaction was stopped by adding $MgCl_2$ to a final concentration of 10 mM.

Isolation of RIP1 cDNA

A ten day whole mouse embryo cDNA expression library (λEXlox, Novagen) was plated on BL21(DE3)pLysE (Novagen) at 10^4 plaques per plate. A total of 2×10^5 plaques were plated. The plates were incubated at 37°C until plaques are about 0.5–1 mm in size and then overlaid with nitrocellulose filters (Schleicher & Schuell) previously saturated in 10 mM IPTG and incubated for an additional 3–5 h at 37°C. The filters were blocked at 4°C in HBB (25 mM HEPES-KOH pH 7.7, 25 mM NaCl, 50 mM $MgCl_2$, 5% milk) 4–10 h and hybridized in Hyb 75 (20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 10 mM $MgCl_2$, 0.05% NP-40) plus 1% dried non-fat milk with $2-7 \times 10^5$ c.p.m. probe per ml of hybridization solution, overnight with rocking at 4°C. The filters were then washed in Hyb 75 and exposed at −80°C for 1–3 h.

Phage from positive plaques were used to infect the BM25.8 strain of *E. coli* to allow the cre-lox mediated excision of the insert containing plasmid, pEXlox, from the λEXlox vector. DNA sequencing was done using a Sequenase kit (Amersham). [^{35}S]dATP for sequencing was from Amersham. Oligonucleotides were synthesized on an Applied Biosystem model 391 DNA synthesizer. A 342 base pair EcoRI-PstI fragment was used as a DNA probe to rescreen the library for full length cDNAs.

Mapping of the interacting domain

Nested deletions were created using Erase-a-base (Promega) and verified by sequencing. 1 μg of plasmid was used per 25 μl *in vitro* translation reaction (TNT coupled Reticulocyte Lysate System, Promega; [^{35}S]-methionine, NEN DuPont). 5 μl were diluted into 1 ml of ELB+ (150 mM NaCl, 0.1% NP-40, 50 mM HEPES, pH 7.4, 5 mM EDTA, 0.5 mM DTT, 1 mM PMSF 10 mM $MgCl_2$) and incubated with HA (hemagglutinin antigen)-tagged human RalB preloaded with GTPγS. The complexes were then immunoprecipitated with anti-Ha antibody, washed with ELB+, electrophoresed on an SDS-10% polyacrylamide gel and exposed overnight at −70°C.

Northern analysis

A 342 base pair EcoRI-PstI fragment was used to probe (Oligolabeling Kit, Pharmacia; [^{32}P]dCTP, NEN DuPont) Multiple Tissue Northern Blots (Clontech).

Expression of recombinant RIP1

The coding region RIP1 was cloned into pGEX-4T (Pharmacia). Transformed bacteria were induced with 0.1 mM IPTG for 4 h, centrifuged and washed. The pellet was resuspended in STE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA) containing 100 $\mu g\ ml^{-1}$ of lysozyme and incubated on ice. DTT was added to final concentration of 5 mM. The bacteria were sonicated and centrifuged. Triton X-100 was added to the supernatant to a final concentration of 2%. Glutathione Sepharose 4B (Pharmacia) beads were added and incubated at 4°C for 30 min. The beads were washed in PBS. RIP1 was cleaved off by adding thrombin in PBS, incubating overnight.

Alternatively, the coding region of RIP1 was also cloned into pVL1393 (Pharmingen). A high titer baculoviral stock was prepared by using the BaculoGold System (Pharmingen) in Sf9 insect cells and used to infect High Five insect cells (Invitrogen). The cells were collected, washed with PBS and resuspended in buffer A (10 mM Tris, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 1 mM EDTA) + protease inhibitors. The cells were lysed in a Dounce homogenizer and centrifuged to pellet nuclei. 0.11 volumes of buffer B (100 mM Tris, pH 7.5, 1500 mM NaCl, 15 mM $MgCl_2$, 100 mM KCl, 50 mM β-mercaptoethanol, 10 mM EDTA, 1% Triton X-100) was added to the supernatant. Following a second centrifugation to pellet cellular debris, Ni-NTA (Qiagen) was added to the supernatant and incubated for 1 h. The beads were then washed with 0.1 × buffer B + 1 mM imidazole. loaded onto a column and washed with buffer B− (0.1 × buffer B minus Triton X-100) plus 16 mM imidazole and 10% glycerol. The protein was eluted in a 10 ml gradient of 16–150 mM imidazole in buffer B−. Positive fractions were dialyzed against buffer B− to remove the imidazole.

GTPases

H-ras and Ral were prepared as previously described (Albright *et al.*, 1993). HA-ral was expressed by cloning the coding region of human RalB downstream of the HA epitope and upstream of 6 histidine codons into pET-15 and purified as described above for KH-ral. Rac1, CDC42, and RhoA were kindly provided by Dr Marc Symons (Malcolm *et al.*, 1994).

Antibody Preparation

The EcoRI-HindIII insert from pEXlox19-6 was cloned into pET-29a (Novagen). Protein was expressed, purified (S-Tag Purification System. Novagen) and used to immunize rabbits. The resulting antiserum was affinity purified against full length GST-RIP1 protein by binding the protein to Glutathione Sepharose 4B (Pharmacia) and washing with 0.1 M borate buffer (pH 8.0). 0.2 M triethanolamine (pH 8.2). The protein was cross-linked to the column by incubation in 40 mM dimethylpimelimidate (DMP) in 0.2 M triethanolamine. The column was then washed in 40 mM ethanolamine (pH 8.2), 0.1 M borate (pH 8.0). The antiserum was passed over the column. Following washes in PBS and PBS + 2 M KCl the antibody was eluted in 5 M NaI and 1 mM sodium thiosulfate in PBS.

Western analysis

Lysates from Balb/3T3 cells and purified RIP1 protein were run on a SDS-7.5% acrylamide gel and transferred to nitrocellulose. The filter was blocked in PBS plus 10% dried non-fat milk for 1 h, incubated with affinity purified anti-RIP1 antibody in PBST (PBS plus 0.1% Tween-20) for 1 h, washed with PBST and incubated with horseradish peroxidase conjugated donkey anti-rabbit IgG antibody

(Jackson ImmunoResearch) in PBST. The filter was washed in PBST and visualized used ECL (Amersham).

GAP Assay

1 μg GTPase was combined with 10 μCi [γ^{32}P]GTP (NEN DuPont) in 100 μl exchange buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA, 1 mg ml^{-1} BSA, 1 mM DTT), 10 min, 25°C. The exchange reaction was stopped by adding 100 μl stop exchange buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 20 mM $MgCl_2$, 5 mM EDTA, 1 mg ml^{-1} BSA, 1 mM DTT), diluted into reaction buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mg ml^{-1} BSA, 1 mM DTT), and aliquoted into 100 μl reactions (10 ng of GTPase per reaction). 0.5 micrograms of RIP1 was added per reaction and incubated 10 min, 25°C. The reaction was diluted in 500 μl of wash buffer (50 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT), filtered through nitrocellulose and washed with additional 10 ml of wash buffer.

For GAP assays in the presence of Ral, 5 micrograms of GTPγS or GDP preloaded Ral was incubated with 0.5 micrograms of RIP1 on ice for 30 min prior to addition to the GAP assay.

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